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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/53, A61K 48/00, C12N 9/06, 5/06 — A1

(11) International Publication Number: WO 97/32025

(43) International Publication Date: 4 September 1997 (04.09.97)

(21) International Application Number: PCT/US97/02772

(22) International Filing Date: 26 February 1997 (26.02.97)

(30) Priority Data:
60/012,677 1 March 1996 (01.03.96) US

(71) Applicant *(for all designated States except US)*: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morrill Hall, 100 Church Street S.E., Minneapolis, MN 55455 (US).

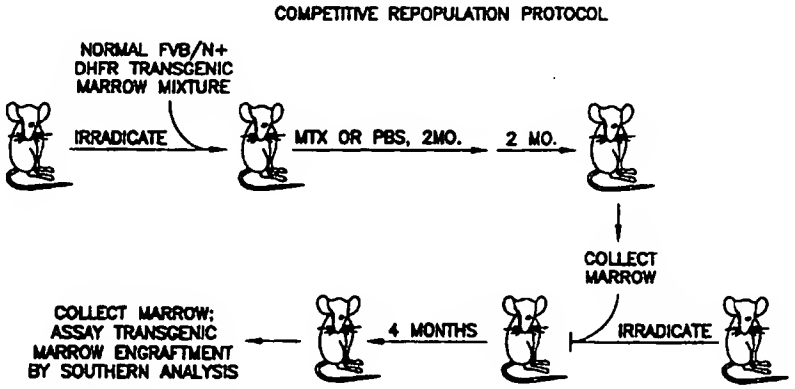
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MCIVOR, R., Scott [US/US]; 3745 Glenhurst Avenue South, St. Louis Park, MN 55416 (US). JAMES, Rohaizah, Ismail [US/US]; 7875 East River Road, Apt. 204, Fridley, MN 55432 (US). MAY, Chad [US/US]; Apartment 827, 445 East 69th Street, New York, NY 10021 (US).

(74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR SELECTIVE ENGRAFTMENT OF DRUG-RESISTANT HEMATOPOIETIC STEM CELLS



(57) Abstract

A method for the selective engraftment of hematopoietic stem cells is provided for increasing the efficiency of hematopoietic stem cell engraftment *in vivo*, comprising: (a) administering to a mammal a population of stem cells, comprising transgenic stem cells, the genome of which has been augmented by a first preselected DNA segment, which encodes resistance to an agent, and which said first preselected DNA segment is operably linked to a promoter functional in stem cells, wherein the expression of the first preselected DNA segment in the transgenic stem cells is sufficient to impart resistance or tolerance to said transgenic cells to an amount of the agent which is toxic to nontransgenic stem cells, the genome of which has not been augmented by the first preselected DNA segment; and (b) administering said agent to said mammal in an amount and for a time so as to increase the engraftment of transgenic stem cells relative to the engraftment of nontransgenic stem cells.

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METHOD FOR SELECTIVE ENGRAFTMENT OF DRUG-RESISTANT HEMATOPOIETIC STEM CELLS

Background of the Invention

As many hereditary diseases are a result of defects in single genes, there are many potential applications of gene therapy in the treatment of single gene disorders. Moreover, gene therapy can also be useful in the treatment of acquired diseases, e.g., cancer and infectious diseases. In particular, many of these diseases can potentially be treated or prevented by the introduction of a therapeutic gene(s) into hematopoietic stem cells (HSC), since the entire hematopoietic system can theoretically be regenerated from a single stem cell.

With a few exceptions (e.g., hormones), most anticancer drugs used in the clinic today cause moderate to severe bone marrow toxicity (e.g., vinblastine, cisplatin, methotrexate, alkylating agents, and anthracyclines). The introduction of a gene that confers resistance to a chemotherapeutic drug (termed a drug resistance gene) into HSC can convert these bone marrow progenitor cells to a drug resistant state, thus allowing larger than conventional doses of chemotherapeutic agents to be administered safely to patients, without toxicity to bone marrow, the gastrointestinal tract, and other normal proliferating tissue.

The first eukaryotic drug resistance gene to be transferred to reconstituting bone marrow cells was a methotrexate (MTX) resistant rodent dihydrofolate reductase (mDHFR) gene. Mice transplanted with cells transduced with a mDHFR containing retroviral vector were protected from methotrexate induced myelosuppression (Corey et al., Blood, 75, 337 (1990); Williams et al., J. Exp. Med., 166, 210 (1987)). Subsequent experiments have suggested that methotrexate can also be used to select for murine hemopoietic cells expressing transferred mDHFR genes (Vinh and McIvor, J. Pharmacol. Exp. Ther., 267, 989 (1993)).

Transfer of the human *mdr*1 gene to hemopoietic cells has also been described. P-glycoprotein, the product of the *mdr*1 gene, functions as a drug efflux pump and confers resistance to a wide variety of naturally occurring

chemotherapeutic agents. Mice transplanted with *mdr*1 transduced cells showed attenuation of taxol induced myelosuppression (Sorrentino et al., Science, 257, 99 (1992); Hanania et al., Blood, 82, 1260 (1990)). In taxol treated animals, the proportion of circulating leukocytes transduced with the *mdr*1 virus increased with drug treatment, suggesting that cells expressing the transferred *mdr*1 gene can be dominantly selected *in vivo* with taxol (Sorrentino et al., *supra*; Podda et al., Proc. Nat'l Acad. Sci. USA, 89, 9676 (1992)). More recent work has suggested that the *mdr*1 gene can be used to select for the presence of other therapeutic genes when the therapeutic genes are linked to the *mdr*1 cDNA in bicistronic retroviral vectors. Thus, drug resistance genes can be used to attenuate drug induced myelosuppression and can act as dominant selectable markers for genetically altered hemopoietic cells.

While HSC are tempting targets for gene transfer, these cells can be transduced with only limited efficiency, since generally less than 0.01% of the cells in bone marrow are HSC. This limits the implementation of clinical protocols based on gene modified HSC. Moreover, even if a therapeutic gene can be stably integrated into HSC, once transferred to a recipient, the transgenic HSC have no selective growth advantage relative to their nontransgenic counterparts. Without such an advantage, the engraftment of HSC containing the therapeutic gene is uncertain and, thus, the curative effect of the expression of the therapeutic gene in HSC and their progeny is unlikely.

One way to increase the representation of successfully transduced HSC would be to mediate selective engraftment by expression of a drug resistance gene. Although selective expansion of hematopoietic cells derived from transduced stem cells has been demonstrated for stem cells transduced with drug resistance genes, the ability of these drug-resistance genes to confer selective engraftment of HSC has not been established by long-term reconstitution studies.

Thus, what is needed is an improved method to select for engraftment of transplanted hematopoietic cells.

Summary of the Invention

The present invention provides a method for selective engraftment of hematopoietic stem cell *in vivo*. The method comprises administering to a mammal a population of stem cells comprising transgenic stem cells, the genome of which has been augmented by a first preselected DNA segment which is operably linked to a promoter functional in stem cells. The first preselected DNA segment encodes resistance to an agent which is normally toxic to stem cells. A preferred embodiment of the invention includes a first preselected DNA segment which encodes resistance to a chemotherapeutic agent, such as an antineoplastic or cytotoxic agent. The genome of the transgenic stem cells can also be augmented by a second preselected DNA segment which encodes a therapeutic agent. The expression of the first preselected DNA segment in the transgenic stem cells is effective to impart resistance or tolerance to said transgenic cells to an amount of the agent which is toxic to the corresponding nontransgenic stem cells. The agent is administered to said mammal in an amount, and for a time, so as to increase the engraftment and proliferation of transgenic stem cells relative to the engraftment and proliferation of nontransgenic stem cells. A preferred embodiment of the invention includes daily administration of the agent.

As used herein, the term "hematopoietic stem cells (HSC)" means a population of primitive progenitor cells which can provide long term reconstitution of both myeloid and lymphoid cell lineages in a host.

As used herein, a cell which is "resistant or tolerant" to an agent means a cell which has been genetically modified so that the cell proliferates in the presence of an amount of an agent that inhibits or prevents proliferation of a cell without the modification.

As used herein, a preselected DNA segment that encodes "resistance" to an agent, such as a preselected DNA segment that encodes resistance to a chemotherapeutic agent, e.g. methotrexate, means that the expression of the preselected DNA segment in a cell permits that cell to proliferate in the presence of the agent to a greater extent than the proliferation of

a corresponding cell without the preselected DNA segment. A preselected DNA segment of the invention can encode resistance to methotrexate, vinblastine, cisplatin, alkylating agents, or anthracyclines, their analogs or derivatives, and the like.

As used herein with respect to an agent, the term "therapeutically effective amount" means an amount of the agent that inhibits or prevents proliferation of untransformed cells in a mammalian host.

As used herein, the term "a preselected DNA segment encoding a therapeutic agent" is defined as a preselected DNA segment encoding any polypeptide, peptide, protein, or sense or antisense RNA that imparts a desired effect to the mammal when it is produced by the cells of said mammal, including, but not limited to, an enzyme (e.g, adenosine deaminase, thymidine kinase, glucose cerebrosidase), a hormone (e.g., human growth hormone, insulin), a cytokine, clotting factors, a hormonal regulator (e.g., amylin, erythropoietin), antisense oligonucleotides (e.g., antisense to the mRNA encoding $P210^{BCR/ABL}$) and the like.

As used herein, the term "engraftment" with respect to HSC means that HSC which are introduced into a recipient are localized in the bone marrow of the recipient and can provide long term reconstitution of both myeloid and lymphoid cell lineages in that recipient.

Brief Description of the Figures

Figure 1 depicts a competitive repopulation protocol to selectively engraft DHFR transgenic marrow into an irradiated recipient. MTX = methotrexate. DHFR = dihydrofolate reductase.

Figure 2 depicts a Southern blot of DNAs isolated from the bone marrow of mice that underwent the competitive protocol shown in Figure 1. DNAs were restricted with BglII. Lane 1 contains marrow DNA from a control female mouse that was transplanted with normal, nontransgenic marrow. Lanes 2-6 contain marrow DNA from five mice that were secondary recipients of marrow from PBS-administered animals that received a primary transplant comprising 50% normal marrow and 50% DHFR transgenic marrow. Lanes 7-

11 contain marrow DNA from secondary recipients of marrow from MTX-administered animals that received a primary transplant comprising 50% normal marrow and 50% DHFR transgenic marrow. Lane 12 contains marrow DNA from a mouse that was a secondary recipient of marrow from an animal that received a primary transplant comprising 100% DHFR transgenic marrow. Lane 13 contains marrow DNA from a control male mouse that did not undergo the protocol. Lane 14 contains marrow DNA from a DHFR transgenic mouse. The position of the endogenous DHFR gene and the introduced transgene is shown at the right.

Detailed Description of the Invention

To improve cancer therapy and selective engraftment of transfused or transplanted tissues, drug resistance genes can be transferred into cells, e.g., HSC, which are normally drug sensitive. The transfer of the drug resistance gene into HSC protects the transplanted transgenic HSC from the toxic side effects of chemotherapy with an antineoplastic or cytotoxic agent. The present method thus allows the administration of higher doses of the chemotherapeutic agent to the host. The transfer of drug resistance genes into HSC also permits the transgenic HSC and their progeny to be selectively enriched *in vivo*, as HSC and their progeny which contain and express the drug resistance gene and other genes which are co-introduced into those cells, will survive and multiply relative to cells which lack the drug resistance gene in the presence of the drug. Although selective expansion of transduced stem cells into more differentiated blood cell types has been previously demonstrated, the selective engraftment of primitive stem cells based on expression of a drug resistance gene has not.

In a preferred embodiment of the invention, the genome of HSC is transformed with a preselected DNA segment which encodes resistance to a chemotherapeutic agent, such as methotrexate, to yield transgenic HSC. A population of HSC which includes the transgenic HSC is then introduced into a recipient mammal. The administration of the chemotherapeutic agent to the recipient inhibits or greatly reduces the proliferation of untransformed HSC

which are undergoing engraftment, while the transgenic HSC which are undergoing engraftment survive and proliferate, i.e., they are resistant to the agent. The proliferation of the engrafted transgenic HSC results in an increase in the relative number of engrafted transgenic HSC, as well as an increase in the number of differentiated progeny which are derived from the engrafted transgenic HSC. These progeny contain the preselected DNA segment and thus are resistant to the chemotherapeutic agent. Transplanted HSC do not significantly contribute to differentiated progeny lineages for at least about 2-4 weeks after the stem cells have been transplanted.

A. Hematopoietic Stem Cells

The human hematopoietic system is populated by a hierarchy of cells with differing capacities for self renewal, proliferation and differentiation. There are cells of several different lineages. These "blood cells" may appear in bone marrow, the thymus, lymphatic tissue(s) and in peripheral blood. Within any specific lineage, there are a number of maturational stages. In most instances, the more immature developmental stages occur within bone marrow while the more mature and final stages of development occur in peripheral blood.

There are two major lineages: The myeloid lineage which matures into red blood cells, granulocytes, monocytes and megakaryocytes; and the lymphoid lineage which matures into B lymphocytes and T lymphocytes. Within each lineage and between each lineage, antigens are expressed differentially on the surface and in the cytoplasm of the cells in a given lineage. The expression of one or more antigens and/or the intensity of expression can be used to distinguish between maturational stages within a lineage and between lineages.

Assignment of a cell to a lineage and to a maturational stage within a cell lineage indicates lineage commitment. There are cells, however, which are uncommitted to any lineage (i.e., "progenitor" cells) and which, therefore, retain the ability to maintain their population and to differentiate into each lineage. These undifferentiated, pluripotent progenitor cells are referred to as the "hematopoietic stem cells (HSC)."

All of mammalian hematopoietic cells can, in theory, be derived from a single stem cell. *In vivo*, the stem cell is able to self-renew, so as to maintain a continuous source of pluripotent cells. In addition, when subject to particular environments and/or factors, the stem cells may differentiate to yield dedicated progenitor cells, which in turn may serve as the ancestor cells to a limited number of blood cell types. These ancestor cells will go through a number of stages before ultimately yielding mature cells.

The benefit of identifying and obtaining a pure population of stem cells is most readily recognized in the field of gene therapy. Gene therapy seeks to replace or repopulate the cells of the hematopoietic system which contain a defective gene with cells that do not contain the defective gene but instead contain a "normal" gene. Thus, using conventional recombinant DNA techniques, a "normal" gene is isolated, placed into a viral or other vector, and the vector is transfected into a cell capable of expressing the product coded for by the gene. The cell then must be introduced into the patient. If the "normal" gene product is produced in sufficient quantity, the patient is "cured" of the condition.

However, the transformed cells must be capable of continual regeneration as well as growth and differentiation. Thus, while Kwok et al. (PNAS USA, 83, 4552 (1986)) demonstrated that gene therapy was possible using retroviral vector-transduced progenitor cells in dogs, the transduced cells were not capable of self-renewal. Thus, the "cure" was only temporary.

Other difficulties encountered in stem cell gene therapy are due to the fact that the stem cell population constitutes only a small percentage of the total number of leukocytes in bone marrow. Weissman et al. (EPO 341,966) reported that murine bone marrow contains only about 0.02-0.1% pluripotent stem cells. Moreover, the introduction of between 20-30 of these stem cells per recipient are necessary to rescue 50% of a group of lethally-irradiated mice. See Weissman et al., *supra* and Spangrude et al., Science, 241, 58 (1988).

The development of cell culture media and conditions to maintain stem cells *in vitro* for the extended periods of time required for the procedures

involved in gene therapy, identification of growth factors, thorough characterization of cell morphologies and the like, has presented a unique set of obstacles. To date, successful *in vitro* stem cell cultures have depended on the ability of the laboratory worker to mimic the conditions which are believed to be responsible for maintaining stem cells *in vivo*.

For example, hematopoiesis occurs within highly dense cellular niches within the bone marrow in the adult and in similar niches within the fetal yolk sac and liver. Within these niches, stem cell differentiation is regulated, in part, through interactions with local mesenchymal cells or stromal cells. Mammalian hematopoiesis has been studied *in vitro* through the use of various long-term marrow culture systems. T.M. Dexter et al., in J. Cell Physiol., 91, 335 (1977) described a murine system from which spleen colony-forming units (CFU-S) and granulocyte/macrophage colony forming units (CFU-GM) could be detected for several months, with erythroid and megakaryocytic precursors appearing for a more limited time. Maintenance of these cultures was dependent on the formation of an adherent stromal cell layer composed of endothelial cells, adipocytes, reticular cells, and macrophages.

These methods were soon adapted for the study of human bone marrow. Human long-term culture system were reported to generate assayable hematopoietic progenitor cells for 8 or 9 weeks, and, later, for up to 20 weeks (See, S. Gartner, et al., PNAS USA, 77, 4756 (1980); F.T. Slovick et al., Exp. Hematol., 12, 327 (1984)). Such cultures were also reliant on the preestablishment of a stromal cell layer which must frequently be reinoculated with large, heterogeneous populations of marrow cells. Hematopoietic stem cells have been shown to home and adhere to this adherent cell multilayer before generating and releasing more committed progenitor cells (M.Y. Gordon et al., J. Cell Physiol., 130, 150 (1987)).

Stromal cells are believed to provide not only a physical matrix on which stem cells reside, but also to produce membrane-contact signals and/or hematopoietic growth factors necessary for stem cell proliferation and differentiation. This heterogenous mixture of cells comprising the adherent cell

layer presents an inherently complex system from which the isolation of discrete variables affecting stem cell growth has proven difficult. Furthermore, growth of stem cells on a stromal layer makes it difficult to recover the hematopoietic cells or their progeny efficiently.

Recently, methods have been developed to culture stem cells effectively *in vitro*, in stromal feeder cell-conditioned medium, with or without added cytokines, as taught in U.S. Patent Nos. 5,436,151 and 5,460,964.

B. Mammalian Gene Transfer

Gene transfer methods used to transform mammalian cells can be classified as physical or biological processes. Physical methods include DNA transfection, lipofection, particle bombardment, microinjection and electroporation. Biological methods include the use of DNA and RNA viral vectors. The main advantage of physical methods is that they are not associated with the pathological or oncogenic processes of viruses. However, they are less precise, often resulting in multiple copy insertions, random integration, disruption of foreign and endogenous gene sequences, and unpredictable expression. For human gene therapy, it is desirable to use an efficient means of precisely inserting a single copy of a preselected gene into the host genome. Viral vectors, and especially retroviral and adenovirus associated viral vectors, have become the most widely used method for inserting genes into human cells. Other viral vectors are derived from poxviruses, Herpes simplex virus I, adenoviruses and adeno-associated viruses. However, any method of introducing the vector into a cell that mediates the integration of that vector into the host cell genome, or that permits the vector to be stably maintained in the host cell in the absence of genomic DNA integration, e.g., autonomous replication, is within the scope of the invention. Most of the current and proposed gene therapy clinical protocols employ retroviral vectors.

Furthermore, a vector useful in the practice of the invention can comprise promoter and/or transcriptional enhancer sequences operably linked to the preselected DNA segment. For example, the β-actin promoter, the phosphoglycerate kinase promoter and many of the retroviral and

retrotransposon long terminal repeats can promote transcription of linked sequences in HSC. Other promoters and/or transcriptinal enhancer elements useful in the practice of the invention are known to those of skill in the art.

Retroviruses are single-stranded RNA viruses which replicate viral RNA into DNA by reverse transcription. Upon replication in the host cell, the viral DNA is inserted into the host chromosome, where it becomes a provirus. Due to their efficiency at integrating into host cells, retroviruses are considered to be among the most promising vectors for human gene therapy. These vectors have a number of properties that lead them to be considered promising for genetic therapy of disease. These include: (1) efficient entry of genetic material present in the vector into cells; (2) an efficient process of entry into target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target to particular cellular subtypes through control of the vector-target cell binding and tissue specific control of gene expression.

Retroviral genomes consist of *cis*-acting and *trans*-acting gene sequences. The *cis* regions include the long terminal repeat (LTR) transcriptional promoter and DNA integration sites, the two primer binding sites required for reverse transcription of DNA from viral RNA, and the packaging signals required for efficient packaging of viral RNA into virions. The LTR is found at both ends of the proviral genome. *Trans*-functions include the proteins encoded by the *gag*, *pol*, and *env* genes, which are located between the LTRs. *Gag* and *pol* encode, respectively, internal viral structural and enzymatic proteins. *Env* encodes the viral glycoprotein which confers infectivity and host range specificity on the virus. A retroviral vector generally consists of *cis* sequences and the replacement of the *trans* sequences with a gene(s) of interest. The *trans* functions can be provided by expression the *trans* sequences in a helper cell or by a helper virus. See U.S. Patent No. 5,354,674 for a discussion of the use of retrotransposon vectors, which are related to retroviral vectors, in mammalian gene transfer. For a more detailed description of the retroviral life cycle, retroviral *cis* and *trans* sequences, as well as retroviral vectors, see RNA Tumor

Viruses: Molecular Biology of Tumor Viruses, Weiss et al. (eds), 2nd ed., Cold Spring Harbor, vols. 1 and 2 (1984).

While previous results indicated that retroviral infection of HSCs is relatively inefficient, most likely due to the quiescent state of a vast majority of HSCs, recent evidence suggests that up to 50% of human steady state bone marrow derived long term culture initiating cells (LTC-IC) can be transduced with a retroviral vector when those cells are pre-incubated with stromal conditioned media (SCM^+) containing IL3 ($IL3^+$) and MIP-1α, and when LTC-IC are cocultured with stromal feeders, FN, or immobilized β1-integrin antibodies during the transduction period. LTC-IC are cells that can initiate and sustain long term bone marrow cultures *in vitro*, and can differentiate into myeloid, B-lymphoid, natural killer cell, and T-cell lineages when induced to differentiate *in vitro* by chemical or physical methods, or *in vivo* by transplant into xenogeneic recipients. Thus, culture conditions can be modified to enhance the transduction of HSCs by retroviral vectors.

The invention will be further described by reference to the following detailed examples.

Example I

Characterization of Mutant DHFR Transgenic Mice

Because hematopoietic stem cells are a relatively quiescent cell population, in general these cells are quite resistant to MTX. The rationale for the administration of MTX immediately post-transplant is based on the idea that stem cells must replicate to some extent during the process of engraftment, making them susceptible to MTX selection at that time. The availability of DHFR transgenic mice could provide a unique resource for quantitative studies of selective engraftment, since these mice can be used as a source of donor marrow in which 100% of the cells contain the drug-resistance gene.

To provide a source of DHFR containing HSC, a series of inbred FVB/N mice were used to make FVB/N transgenic mice which express the arg22, trp31 or tyr22 murine DHFR variants, which confer resistance to MTX. MTX resistant variants of mammalian DHFRs have been generated by cloning

DHFR genes from MTX resistance cell lines, and by site directed mutagenesis. MTX resistant variants of dihydrofolate reductase (DHFR) useful in the practice of the invention include, but are not limited to, SER^{31}-DHFR, ASN^{31}-DHFR, HIS^{31}-DHFR, SER^{34}-DHFR, TYR^{22}-DHFR, TRP^{31}-DHF, ARG^{22}-DHFR, TRP^{22}-DHFR, and PHE^{22}-DHFR (see Morris and McIvor, Biochem. Pharmacol., 47, 1207 (1994)).

Line 03 (trp31) and line 04 (arg22) DHFR transgenic mice were found to be more tolerant to MTX than normal animals after sublethal irradiation and daily drug administration at levels of MTX which were 25% the daily dose shown to be lethal for normal FVB/N mice (0.25 mg/kg/day for 1-4 days, 0.5 mg/kg/day for the next four days, then 1.0 mg/kg/day for the remaining time).

Transplantation of 10^6 bone marrow cells from line 04 DHFR transgenic mice into normal, irradiated mice rendered the recipient animals resistant to MTX at levels of methotrexate which caused hematopoietic toxicity in bone marrow recipients that received non-transgenic marrow (0.25 mg/kg/day for 1-4 days, 0.5 mg/kg/day for the next four days, then 1.0 mg/kg/day for the remaining time). Although marrow from line 03 was found to protect transplant recipients from lethal, low-dose MTX toxicity, line 03 marrow was not as effective as line 04 marrow in protecting recipient animals.

Transplantation of 10^7 cells from either line 03 or line 04 DHFR transgenic marrow conferred upon normal irradiated recipient animals resistance to a high dose of MTX (final of 4 mg/kg/day), a level which causes not only hematopoietic toxicity but gastrointestinal toxicity as well. Surprisingly, gastrointestinal cells in animals transplanted with DHFR transgenic marrow were significantly less affected than control animals by the high dose of MTX, indicating that the protective effect of the transgene was systemic, i.e., proliferating tissue which did not contain the transgene was nevertheless protected by the presence of the transgenic HSC. Therefore, drug resistant DHFR expression in hematopoietic cells can provide significant protection from MTX toxicity in animals.

These results suggest a key role for hematopoietic cells in mediating the sensitivity or resistance of animals to MTX. Thus, chemoprotection by MTX-resistant DHFR gene transfer might not be limited to special circumstances that render hematopoietic tissues particularly sensitive to MTX such as extreme cytoreduction and bone marrow transplantation. The present method may be used to permit irradication of MTX-sensitive tumors not usually treated by total body irradiation plus bone marrow transplantation, and for which MTX toxicity to normal tissues limits the utility of MTX.

Example II

Competitive Repopulation of Recipients with Normal and DHFR Transgenic Marrow

A. Competitive Repopulation with 50/50 Mixtures of Normal and Transgenic Marrow at high dose (4 mg/kg/day) MTX

Mice which were transplanted with 10^7 DHFR transgenic marrow cells were protected from the lethal MTX toxicity observed in animals transplanted with 10^7 normal FVB/N donor marrow cells (Example I). Although the ability of a donor marrow population to confer drug-resistance does not necessarily imply that this population is capable of selective engraftment under similar conditions, *in vivo* methotrexate selection may be effective to engraft MTX-resistant transgenic stem cells since there may be sufficient proliferative activity during engraftment to prevent nontransgenic stem cells from engrafting.

To determine if DHFR expression in donor marrow allows selective engraftment in transplant recipients, normal female FVB/N animals were administered a lethal dose of Cs irradiation (900 rads) and then transplanted with approximately 10^7 donor marrow cells consisting of (i) 100% normal male FVB/N marrow, (ii) 100% female line 04 DHFR transgenic marrow (or marrow from another mutant DHFR transgenic line), or (iii) a 50/50 mixture of normal male FVB/N marrow and female DHFR transgenic marrow. Male marrow is used as competitor so that Y chromosome sequences can be used as a quantitative signal for the presence of competitor-derived material at the time of

analysis, while the DHFR transgene can be used as a quantitative signal for presence of DHFR-transgenic-derived material.

Animals in each of the three groups were administered either PBS or MTX at an increasing dose schedule culminating at 4 mg/kg/day. MTX administration was continued out to 60 days post-transplant. Under these conditions of MTX administration, all animals transplanted with FVB/N marrow and administered MTX succumb to MTX induced toxicity at 15-30 days post-transplant. After 60 days, MTX administration is stopped. A small number of animals were sacrificed at this time to collect marrow samples for subsequent analysis, but most of the animals were maintained for an additional 2 months. At 4 months post-transplant, all surviving animals were sacrificed and bone marrow harvested. Each marrow sample collected was resuspended and then injected into 5 lethally-irradiated secondary female FVB/N recipients (2×10^6 donor cells per secondary recipient), saving the leftover marrow for subsequent molecular analysis. Secondary recipients were maintained for an additional 4 months before sacrificing and harvesting marrow samples.

To ensure that all of the marrow cells in the tested sample are derived from primitive, hematopoietic stem cells contained in the original inoculum, experiments are conducted for a full eight months and include transplantation to a secondary recipient. These precautions are necessary because the life span of more differentiated transgenic marrow cells in the original inoculum is from several days up to 2-3 months.

DNA was extracted from bone marrow samples, digested with BglI, electrophoresed on agarose gels and then blotted onto Nytran. Each blot was first probed for DHFR sequences as previously described by May et al. (Blood, 86, 2439 (1995)), and then the blot is stripped and reprobed for Y chromosome sequences using the plasmid pY2 (kindly provided by Dr. Ihor Lemischka, Princeton University).

After hybridization with each probe, the radioactivity associated with DHFR or Y sequences was quantitated using a Molecular Dynamics Phosphorimager. Controls of normal FVB/N male DNA, normal FVB/N female

DNA, and DHFR transgenic female DNA were included on each blot to allow quantitation of relative cellular representation, using the signal from the endogenous DHFR gene as a loading control. The contribution of DHFR transgenic marrow was quantitated as the ratio of the DHFR transgene signal strength to endogenous DHFR gene signal, relative to the same ratio for the 100% control (i.e., the DHFR transgenic female sample). The contribution of competitor marrow was quantitated as the ratio of Y sequence signal strength to endogenous DHFR gene signal, relative to the same ratio for the normal FVB/N male control. The two signals do not always add up to 100%, since radiation-resistant host-derived material can contribute to the ultimate marrow cellularity, and in this case would contribute to the endogenous DHFR gene signal but not to either the Y signal or the DHFR transgene signal. Quantitation of several samples (1 primary and 6 secondary) from each marrow transplant allows assessment of the statistical significance of the results.

Only 2 out of 5 secondary recipients of PBS-administered animals showed a significant contribution of transgenic marrow, while all 5 out of 5 secondary recipients of MTX-administered animals showed a substantial contribution of transgenic marrow, i.e., approximately that observed from injection of 100% transgenic marrow in the primary recipients (Figure 2). The presence and expression of the drug-resistance gene in the transgenic marrow provided the transplanted transgenic marrow with a selective advantage over the normal marrow in establishing a bone marrow graft in the primary recipients. Moreover, animals transplanted with transgenic marrow exhibit a substantial increase in DHFR transgene copy number in primary recipients that had been administered MTX rather than PBS. Therefore, MTX can be used for selective engraftment of stem cells expressing a MTX-resistant DHFR, which leads to an increase in the relative number of MTX-resistant cells in hematopoietic tissues.

B. Competitive repopulation at low dose (1 mg/kg/day) MTX.

Transplantation of 10^6 DHFR transgenic marrow cells protected recipient mice from MTX toxicity at levels up to 1 mg/kg/day (Example 1). Thus, by decreasing the size of the graft, the level of MTX which is tolerated by

the recipient animal can be decreased. To determine if MTX administration at a lower dose along with transplantation at a lower cell dose more effectively provides for selective engraftment of DHFR transgenic stem cells, female FVB/N animals are given a lethal dose of total body irradiation (900 rads cesium) and then transplanted with a total of 10^6 donor marrow cells consisting of the same groups described above, i.e., (i) 100% normal male FVB/N marrow, (ii) 100% female line 04 DHFR transgenic marrow (or marrow from another mutant DHFR transgenic line), or (iii) a 50/50 mixture of normal male FVB/N marrow and female DHFR transgenic marrow.

These animals are administered either PBS or MTX at an increasing dose schedule culminating at 1 mg/kg/day for 60 days. The animals will be maintained until 4 months post-transplant, sacrificed for marrow harvest, and the marrow used for secondary transplants at a dose of 2×10^6 donor cells. Secondary transplant recipients are maintained for 4 months and then sacrificed to collect marrow cells for quantitative Southern hybridization analysis as described above.

A significant increase in the contribution of DHFR transgene sequences associated with MTX administration in animals transplanted with a 50/50 mixture of normal male FVB/N and DHFR transgenic marrow is evidence for selective engraftment of DHFR transgenic stem cells under the conditions used for this experiment (i.e., 10^6 cells transplanted, MTX administered at 1 mg/kg/day).

C. Competitive repopulation of reduced proportions of DHFR transgenic marrow cells.

The competitive repopulation experiments described above test for MTX-mediated selective engraftment of DHFR transgenic marrow cells after transplantation of a 50/50 mixture of normal and transgenic marrow. However, transplantation with a smaller proportion of DHFR transgenic marrow cells may be necessary in order to assess a significant MTX-mediated increase in the contribution of these cells to hematopoiesis in primary or secondary recipients. To determine whether a smaller contribution of transgenic marrow cells can

contribute to engraftment in secondary recipients, animals are irradiated and then transplanted with 10^6 or 10^7 donor marrow cells consisting of the following ratios of DHFR transgenic marrow to normal male FVB/N marrow; 50/50, 20/80, and 5/95. Animals transplanted with 10^6 donor marrow cells are administered PBS, or MTX at a final dose of 1 mg/kg/day, and animals transplanted with 10^7 cells are administered a final dose of 4 mg/kg/day. MTX is administered for 60 days, then animals are maintained without injections for another two months before sacrifice and transplantation into secondary lethally-irradiated recipients (5 secondary recipients per primary recipient, 2×10^6 donor cells per animal). Secondary recipients are maintained for 4 months before sacrifice and collection of marrow for determination of relative donor marrow contribution by Southern hybridization analysis as described above.

Any significant increase in the relative contribution of DHFR transgenic marrow observed for MTX administered animals versus PBS administered animals is evidence for selective engraftment of DHFR transgenic marrow under the conditions tested (with respect to the number of cells transplanted, the dose of MTX administered, and the ratio of DHFR transgenic marrow cells to normal male FVB/N competitor cells).

D. Conclusions

Thus, the method of the present invention can enrich for transgenic stem cells and selectively engraft those stem cells after transplantation even when the transgenic cells comprise a low proportion of the transplanted donor cell population. This approach is clinically useful as a way to improve the representation of transgenic hematopoietic cells post-transplant in spite of an initially low transformation frequency. The result is that the successfully transformed stem cells can rapidly contribute a much greater fraction of the marrow cells in the recipient patient than they do to the original fraction of transformed marrow cells, thus effectively counteracting the low frequency of gene transfer. The invention described herein is thus applicable to any gene therapy protocol which targets hematopoietic stem cells, through the preparation of gene delivery vehicles which contain not only a preselected DNA segment

encoding a therapeutic molecule, but also contain a preselected DNA segment encoding resistance to an agent. Such a protocol can include the daily administration of the agent, wherein the administration begins soon, or immediately, after the transformed cells are transplanted into a recipient.

The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A method for increasing the efficiency of hematopoietic stem cell engraftment *in vivo*, comprising:

(a) administering to a mammal a population of stem cells, comprising transgenic stem cells, the genome of which has been augmented by a first preselected DNA segment, which encodes resistance to an agent, and which said first preselected DNA segment is operably linked to a promoter functional in stem cells, wherein the expression of the first preselected DNA segment in the transgenic stem cells is sufficient to impart resistance or tolerance to said transgenic cells to an amount of the agent which is toxic to nontransgenic stem cells, the genome of which has not been augmented by the first preselected DNA segment; and

(b) administering said agent to said mammal in an amount and for a time so as to increase the engraftment of transgenic stem cells relative to the engraftment of nontransgenic stem cells.

2. The method of claim 1 further comprising augmenting the genome of the transgenic stem cells with a second preselected DNA segment, which encodes a therapeutic agent, and which is linked to the first preselected DNA segment.

3. The method of claim 1 wherein the first preselected DNA segment encodes a methotrexate resistant dihydrofolate reductase.

4. The method of claim 3 wherein the dihydrofolate reductase is SER^{31}-DHFR.

5. The method of claim 3 wherein the dihydrofolate reductase is SER^{34}-DHFR.

6. The method of claim 3 wherein the dihydrofolate reductase is TYR^{22}-DHFR.

7. The method of claim 3 wherein the dihydrofolate reductase is TRP^{31}-DHFR.

8. The method of claim 3 wherein the dihydrofolate reductase is ARG^{22}-DHFR.

9. The method of claim 3 wherein the dihydrofolate reductase is PHE^{22}-DHFR.

10. The method of claim 3 wherein the dihydrofolate reductase is HIS^{31}-DHFR.

11. The method of claim 3 wherein the dihydrofolate reductase is TRP^{22}-DHFR.

12. The method of claim 3 wherein the dihydrofolate reductase is ASN^{31}-DHFR.

13. The method of claim 1 wherein the first preselected DNA segment is the human *mdr*1 gene.

14. The method of claim 1 wherein the transgenic engrafted cells are maintained in the mammal and impart resistance to the agent.

15. The method of claim 1 wherein the administration of the agent begins within 48 hours of stem cell administration.

16. The method of claim 1 wherein the mammal is a human.

17. The method of claim 1 wherein the mammal is subjected to chemotherapy or radiation therapy prior or subsequent to the administration of the population of stem cells.

18. The method of claim 1 wherein the first preselected DNA segment encodes resistance to an antineoplastic agent.

19. The method of claim 18 wherein the antineoplastic agent is methotrexate.

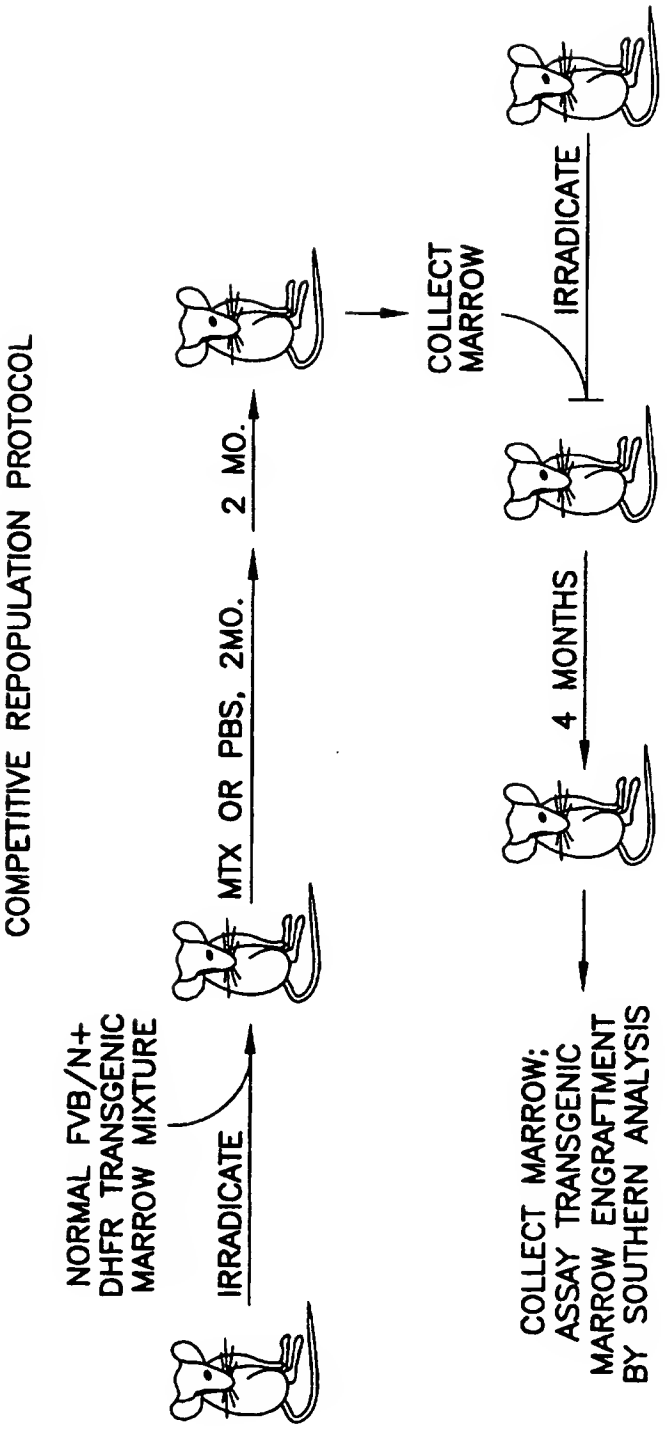
COMPETITIVE REPOPULATION PROTOCOL
NORMAL FVB/N+
DHFR TRANSGENIC
MARROW MIXTURE
IRRADICATE
MTX OR PBS, 2MO.
2 MO.
COLLECT
MARROW
IRRADICATE
4 MONTHS
COLLECT MARROW;
ASSAY TRANSGENIC
MARROW ENGRAFTMENT
BY SOUTHERN ANALYSIS

FIG. 1

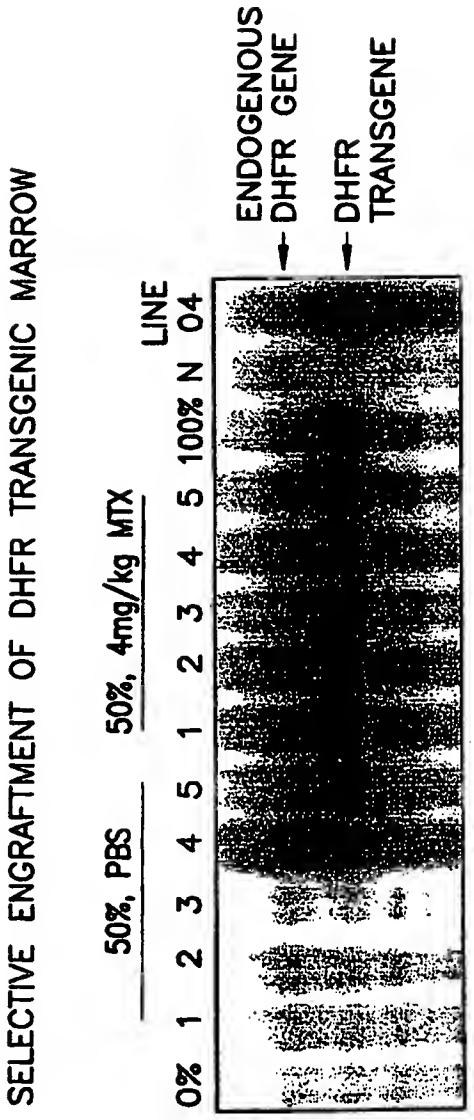
SELECTIVE ENGRAFTMENT OF DHFR TRANSGENIC MARROW
50%, PBS
50%, 4mg/kg MTX
LINE
0% 1 2 3 4 5 1 2 3 4 5 100% N 04
ENDOGENOUS DHFR GENE
DHFR TRANSGENE

FIG. 2

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/02772

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 A61K48/00 C12N9/06 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLOOD, vol. 75, no. 2, 1990, pages 337-343, XP000675196 COREY, C.A. ET AL.: "Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells" see the whole document --- -/--	1-3,14, 15,17-19

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 1997	27.06.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

rnational application No.
PCT/US 97/02772

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see continuation-sheet

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

Remark: Although all the claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition (i.e. transgenic stem cells for a DNA segment which encodes resistance to an agent operably linked to a promoter functional in stem cells).

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/US 97/02772

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLOOD, vol. 86, no. 6, 15 September 1995, pages 2439-2448, XP000675197 MAY, C. ET AL.: "Protection of mice from lethal doses of methotrexate by transplantation with transgenic marrow wxpressing drug-resistant Dihydrofolate Reductase activity" see the whole document ---	1,3,7,8, 14,15, 17-19
X	BLOOD, vol. 83, no. 11, 1 June 1994, pages 3403-3408, XP000675195 LI, M-X. ET AL.: "Development of a retroviral construct containing a human mutated dihydrofolate reductase cDNA for hematopoietic stem cell transduction" see the whole document ---	1-4,14, 17-19
X	LEUKEMIA, vol. 9, no. 1, October 1995, pages S34-S37, XP000675184 FLASSHOVE, M. ET AL.: "Increased resistance to methotrexate in human hematopoietic cells after gene transfer of the Ser31 DHFR mutant" see the whole document -----	1-4,14, 16,18,19

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)